DIRECT AND INDIRECT SOMATIC EMBRYOGENESIS ON ARABICA COFFEE (*Coffea arabica*)

*Embriogenesis Somatik Langsung dan Tidak Langsung pada Kopi Arabika (*Coffea arabica*)*

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ABSTRACT

Propagation of *Coffea arabica* L. through direct and indirect somatic embryogenesis technique is promising for producing large number of coffee seedlings. The objectives of the research were to evaluate methods for direct and indirect somatic embryogenesis induction of *C. arabica* var. Kartika. The explants were the youngest fully expanded leaves of arabica coffee. The evaluated medium was modified Murashige and Skoog (MS) medium supplemented with a combination of 2.26 µM 2,4-D + 4.54 or 9.08 µM thidiazuron; 4.52 µM 2,4-D + 4.54 or 9.08 µM thidiazuron; or 9.04 µM 2,4-D + 9.08 µM thidiazuron. Both calli (100 mg) and pre-embryos developed from the edge of leaf explants were subcultured into regeneration medium (half strength MS with modified vitamin, supplemented with kinetine 9.30 µM and adenine sulfate 40 mg L^{-1}). The results showed coffee leaf explant cultured on medium containing 2.26 µM 2,4-D + 4.54 or 9.08 µM thidiazuron to induce direct somatic embriogenesis from explant, while that of 4.52 or 9.04 µM 2,4-D + 9.08 µM thidiazuron to induced indirect somatic embriogenesis. The medium for calli induction from coffee by explants was medium supplemented with 4.52 or 9.04 µM 2,4-D in combination with 9.08 µM thidiazuron. On the other hand, the best medium for activation of induction of somatic embryos was MS medium supplemented with 9.04 µM 2,4-D + 9.08 µM thidiazuron. Based on this results, the first step for developing micropropagation for coffee has been resolved. The subsequent studies will be directed to evaluate agronomic performance of the derived planting materials.

[***Keywords*****:** *Coffea arabica*, somatic embryogenesis, 2,4-dichlorophenoxyacetic acid, thidiazuron]

ABSTRAK

Perbanyakan Coffea arabica *L. menggunakan teknik embriogenesis somatik secara langsung maupun tidak langsung memberikan harapan dalam penyediaan bibit dalam jumlah besar. Penelitian bertujuan untuk mengevaluasi metode induksi embriogenesis somatik langsung dan tidak langsung dari* C. arabica *var. Kartika. Eksplan yang digunakan adalah daun muda kopi arabika yang sudah membuka sempurna. Media yang dievaluasi adalah media Murashige and Skoog (MS) yang dimodifikasi dan dilengkapi kombinasi 2,4-D 2,26 µM + thidiazuron 4,54 atau 9,08 µM; 2,4-D 4,52 µM + thidiazuron 4,54 atau 9,08 µM; dan 2,4-D 9,04 µM + thidiazuron 9,08 µM. Kalus (100 mg) dan pro-embrio yang berkembang dari pinggir eksplan daun disubkultur ke media regenerasi (MS setengah konsentrasi dengan vitamin yang modifikasi dan dilengkapi kinetine 9,30 µM dan adenin sulfat 40 mg L^{-1}). Hasil penelitian menunjukkan eksplan daun kopi yang dikulturkan pada media yang mengandung 2,4-D 2,26 µM + thidiazuron 4,54 atau 9,08 µM dapat menginduksi embriogenesis somatik langsung, sedangkan media 2,4-D 4,52 atau 9,04 µM + thidiazuron 9,08 µM untuk meginduksi embriogenesis somatik tidak langsung. Media untuk menginduksi kalus kopi adalah media yang dilengkapi dengan 2,4-D 4,52 atau 9,04 µM yang dikombinasikan dengan thidiazuron 9,08 µM. Dengan perkataan lain, media terbaik untuk mengaktivasi induksi embrio somatik adalah media MS yang dilengkapi 2,4-D 9,04 µM + thidiazuron 9,08 µM. Berdasarkan penelitian ini, langkah pertama untuk mengembangkan perbanyakan kopi telah diperoleh. Penelitian selanjutnya akan diarahkan untuk mengevaluasi penampilan agronomi dari bahan tanaman yang dikulturkan.*

[***Kata kunci*****:** *Coffea arabica*, embriogenesis somatik, *2,4-dichlorophenoxyacetic acid*, thidiazuron]

INTRODUCTION

Arabica coffee bean produced from *Coffea arabica* plant is one of the most highly valuable beans used for beverage in more than 50 countries in the world. Multiplication of the superior plant by using conventional vegetative propagation method is genetically stable, but it is very slow to produce large number of

coffee seedlings required by farmers. Therefore, *in vitro* culture techniques such as organogenesis and embryogenesis which have been used in multiplication of various crops are good chances for mass propagation of coffee planting materials.

Multiplication of arabica coffee using organogenesis had low efficiency because of several technical difficulties such as explant sterilization, high concentration of phenol in the explant, apical dominancy, and low rate of shoot multiplication (Raghramuhu *et al.* 1989; Ribeiro and Carneiro 1989). Somatic embryogenesis, on the other hands, was more favorable due to its potential to produce large number of seedlings with lower production cost (Dehayes 2000; Etienne 2005; Kumar *et al.* 2006).

Over the past 40 years, a number of studies on somatic embryogenesis have been developed for producing various coffee genotypes. Somatic embryogenesis of coffee was first reported by Staritsky (1970), followed by others including Etienne *et al.* (2002), Figueroa-Quiroz *et al.* (2002), Oktavia *et al.* (2003), Priyono (2004), Giridhar *et al.* (2004), Albarra'n *et al.* (2005), Samson *et al.* (2006), Andrés *et al.* (2008) and Arimarsetiowati (2011). These studies showed that somatic embryogenesis on coffee still faced obstacles because it is highly dependent on the genotype.

Somatic embryogenesis is a process of developing somatic cells to form new plants through specific stages of embryonic development without going through the fusion of gametes and without vascular connection with the original tissue (Williams and Maheswara 1986; von Arnorld *et al.* 2002). Seed of somatic embryo is a bipolar structure resembling a zygotic embryo development; therefore, plant propagation through somatic embryo formation is more favorable than using organogenesis which produce unipolar seed. Regeneration through somatic embryogenesis in modern biotechnology can be used not only for plant propagation, but also for stabilizing plant genetic because the plant is derived from somatic cell.

Somatic embryogenesis on coffee plants can be divided into direct and indirect embryogenesis. Direct somatic embryogenesis occurs when embryos are started directly from explant tissues (pre-embryogenic cells) creating an identical clone, while indirect somatic embryogenesis occurs from unorganized tissues (calli) which are further developed into embryos (Nakamura *et al.* 1992; Calheiros *et al.* 1994). Formation of embryo through direct embryogenesis is preferred than the indirect one because it can avoid a problem in forming seed in the somatic germination stage (Rai and Mccomb 2002) and limit the occurrence of somalonal variation. However, the number of somaclones produced through direct embryogenesis is usually limited and not uniform; therefore indirect somatic embryogenesis is used for improvement and propagation of crops.

The success of regeneration through somatic embryogenesis is influenced by several factors, including physiological conditions of the plant and types of media formulation used which lead to low reproducible rate. This research aimed to study the regeneration system of *C. arabica* var. Kartika through direct and indirect somatic embryogenesis. The method is expected to be used for propagation of arabica coffee planting materials.

MATERIALS AND METHODS

Plant Materials and Explant Preparation

Arabica coffee var. Kartika used in the study originated from the germplasm collection of the Indonesian Research Institute for Industrial and Beverages Crops at Sukabumi, West Java. The plant was grown in a greenhouse of the Agricultural Superior Seed Development Unit, Indonesian Agency for Agricultural Research and Development. Most recently formed and fully expanded leaves of the plant were used as explants. The explants were cleaned with running water for 10 minutes, soaked in 0.2% fungicide solution containing active ingredient of mancozeb 80% for one hour and rinsed with distilled water to clean fungicide excess. The treated leaves were then aseptically surface sterilized by dipping in ethanol 70% for 3 minutes followed in hypochlorite 10% for 15 minutes in a laminar air flow cabinet. The treated leaves were rinsed three times in sterile distilled water, cut into 1 cm x 1 cm sized and placed in a sterile petridish.

Callus Induction

A modified Murashige and Skoog medium containing ammonium nitrate was used for calli induction (Samson *et al.* 2006). Plant growth regulators 2,4-dichlorophenoxyacetic acid (2,4-D) and thidiazuron were added as treatments. Five treatments tested were (1) 2,4-D 2.26 µM + thidiazuron 4.54 µM, (2) 2,4-D 2.26 µM + thidiazuron 9.08 µM, (3) 2,4-D 4.52 µM + thidiazuron 4.54 µM, (4) 2,4-D 4.52 µM + thidiazuron 9.08 µM, and (5) 2,4-D 9.04 µM + thidiazuron 9.08 µM.

The callus induction medium was enriched with sucrose (30 mg L^{-1}) and polynil polypyrolidon (PVPP; 250 mg L^{-1}) and the pH was adjusted to 5.6 ± 0.1 by adding 1 M NaOH. The medium was solidified with phytagel (2.5 g L^{-1}) and sterilized by autoclaving at 121°C for 15 minutes. The sterilized medium was then poured into 6.0 cm diameter glass bottles and the sterilized leaf explants were placed on the callus induction medium according to the treatments. The cultures were incubated in a dark condition at 25 ± 2°C for 1 month.

Embryogenic Callus Induction

After one month in the callus induction medium, the explants were subcultured into the advanced callus induction medium containing half strength of MS medium supplemented with macro- and microsalts, i.e. thiamine (20 mg L^{-1}), glycine (20 mg L^{-1}), niacin (1 mg L^{-1}), myoinositol (200 mg L^{-1}), L-cysteine (40 mg L^{-1}), casein hydrolysate (200 mg L^{-1}), malt extract (800 mg L^{-1}), 2,4-D (4.52 µM), BA (17.76 µM), adenine sulfate (60 mg L^{-1}), sucrose (30 g L^{-1}) and phytagel (2.5 g L^{-1}) as solidified agent based on van Boxtel and Berthouly (1996) and Etienne (2005). The cultures were incubated in a dark condition at 25 ± 2°C for 3-4 months.

The experiment was arranged in a completely randomized design with eight replications. Each replication consisted of one bottle containing five explants. The variables measured were percentage of calli formation and fresh weight of culture. Data were analyzed statistically using the SAS 9.1 program. If there is a significant difference, the data were further analyzed by using Duncan's Multiple Range Test (DMRT) at 5% level.

Somatic Embryo Development

Indirect Somatic Embryogenesis

Calli produced from the leaf tissue on callus induction medium were separated and placed in a sterile petridish. The embryogenic calli were weighed (100 mg) and subcultured on the regeneration medium containing a half strength of MS supplemented with macro- and microsalts, thiamine (10 mg L^{-1}), glycine (20 mg L^{-1}), niacin (1 mg L^{-1}), myoinositol (200 mg L^{-1}), L-cysteine (10 mg L^{-1}), casein hydrolysate (400 mg L^{-1}), malt extract (400 mg L^{-1}), kinetine (9.30 µM), adenine sulfate (40 mg L^{-1}) and sucrose (35 g L^{-1}), as well as phytagel (2,5 g L^{-1}) to solidified the medium (van Boxtel and Berthouly 1996).

Direct Somatic Embryogenesis

Explants that have formed pre-embryos (direct embryogenesis) on calli induction medium were transferred to the regeneration medium. Composition of the regeneration medium was the same with that used for indirect somatic embryogenesis as described before.

Variables observed, both in the indirect and direct somatic embryogenesis were the number of globular and torpedo somatic embryos.

Experimental Design and Data Analyses

The experiment was arranged in a completely randomized design with ten replications. Data collected were presented as means and standards of deviation and statistically analysed using the SAS 9.1 program. If there is a significant difference, the data were further analyzed by using Duncan's Multiple Range Test (DMRT) at 5% level.

RESULTS AND DISCUSSION

Callus Induction

Three weeks after incubation on callus induction medium, the coffee leaf tissues started to proliferate at the incision site of the leaf. At the 4th week, the calli were formed (Fig. 1a). Fresh weights of the explants after one month incubation on callus induction medium were shown in Figure 2. Increase in explant fresh weights correlated with the addition of plant growth regulators of 2,4-D and thidiozuron. The highest explant fresh weight was found on MS medium treated with a combination of 2,4-D (9.04 µM) and thidiazuron (9.08 µM). This result was similar with previous studies conducted by Ibrahim *et al.* (2012) and Ibrahim *et al.* (2013) although they used different combinations and concentrations of plant growth regulators, i.e. 2,4-D + kinetine or 2,4-D + BA. Increase in pre-embryogenic calli was due to alleviation of cell division to produce pre-embryogenic mass.

Embryogenic Callus Induction

Percentage of calli formed on the second callus induction medium varied. The lowest callus percentage was obtained on MS medium treated with 2,4-D (2.26 µM) + thidiazuron (4.54 µM or 9.08 µM)

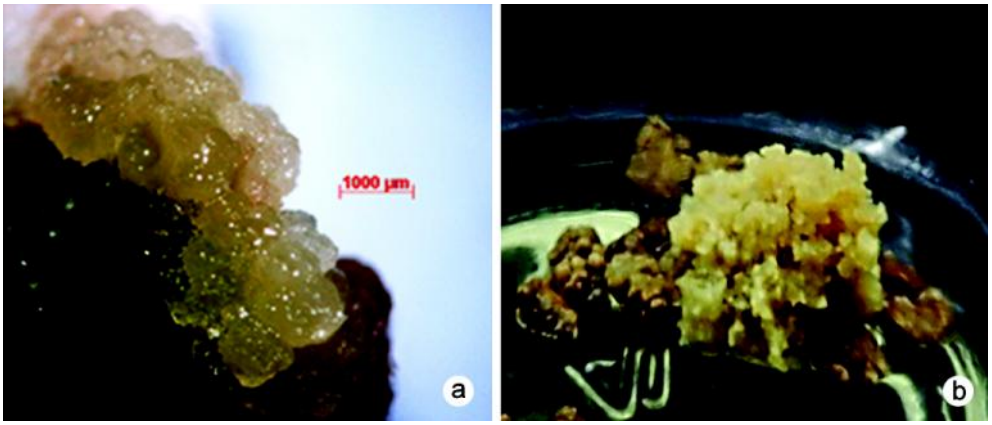

Fig 1. Calli of arabica coffee on induction medium containing 2,4-D 9.04 µM + thidiazuron 9.08 µM at one month after aplication (a) and embryogenic calli on induction medium containing 2,4-D 9.04 µM + thidiazuron 9.08 µM (b).

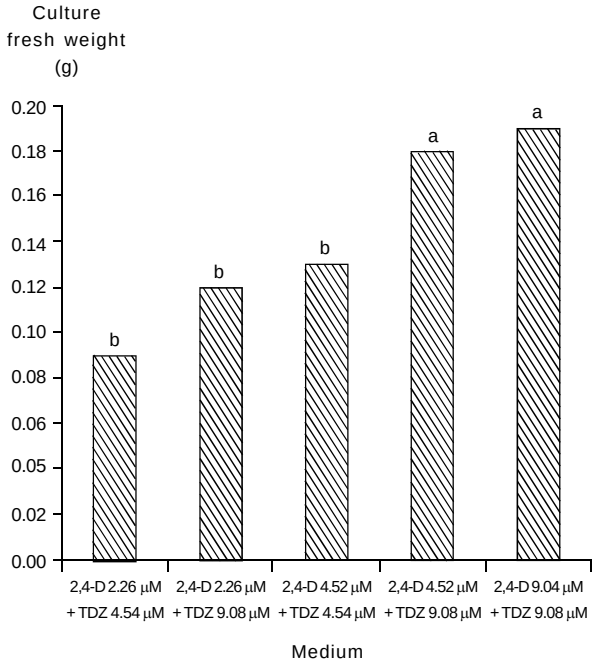

Fig 2. Culture fresh-weight of arabica coffee at one month on calli induction medium. The same letters on top of the bar indicate the treatment was not significantly different at α = 0,05. TDZ = thidiazuron.

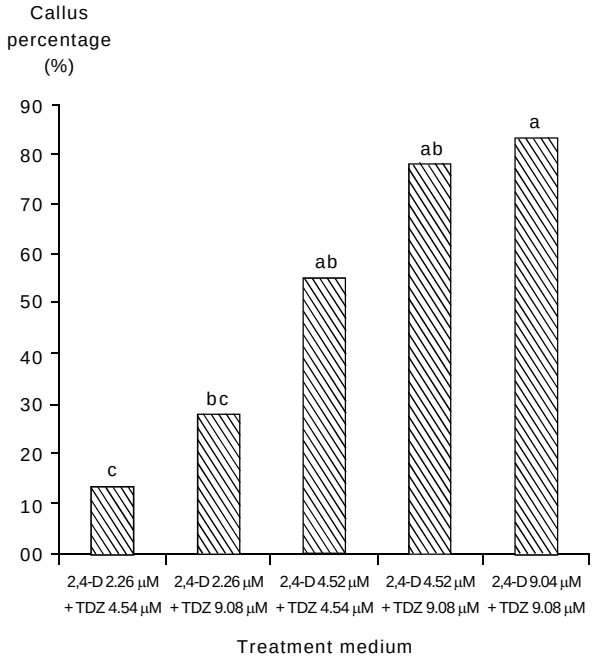

Fig. 3. Percentage of calli of arabica coffee at three months after culture on advanced callus induction medium. The same letters on top of the bar indicate the treatment was not significantly different at α = 0,05. TDZ = thidiazuron.

(Fig. 3). The compact nodular nonembryogenic calli developed on the cut edge of the leaf, but the calli did not show a continuous growth. Morphological observation using a stereo microscope showed that pre-embryos were formed along the incision site of the leaf indicating a direct embryogenesis process of the leaf. The nonembryogenic calli were similar with that reported by Gatica *et al.* (2007) who obtained low frequency of callus formation in inducing direct somatic embryogenesis of arabica coffee cv. Catura and Catuai (Gatica *et al*. 2008). In the direct embryogenesis, calli were produced on MS medium treated with 2,4-D (4.52 µM or 9.04 µM) + thidiazuron (9.08 µM). It was interesting to note that both direct and indirect somatic embryogenesis produced calli from treatment of 2,4-D (4.52 µM) + thidiazuron (4.54 µM). The highest percentage of calli was found on the treatment of 2,4-D (9.04 µM) + thidiazuron (9.08 µM).

This result showed that morphogenesis on coffee tissue culture was highly dependent on the ratio of auxin and cytokinin in the callus induction medium. Calli were formed if the ratio of auxin and cytokinin was higher (George and Sherrington 1984; Tores 1989).

Somatic Embryo Development

Indirect Somatic Embryogenesis

The development of embryos through indirect somatic embryogenesis on the coffee tissues was characterized by small, yellowish and dense cytoplasm with large nuclei and vacuoles structures containing grains starch (Williams and Maheswaran 1986) (Fig. 1b). The color of the embryogenic calli changed from yellow to brownish black after grown on regeneration medium for two months. Under observation with an electron microscope, the three months old calli appeared blackish brown and white pre-embryo. After four months in regeneration medium, the pre-embryo developed into globular and to be torpedo on the eight months. The study showed that indirect embryogenesis produced more globular and torpedo embryos (Fig. 4).

The highest number of globular and torpedo embryonic calli were found on the treatment of 2,4-D 9.04 µM + thidiazuron 9.08 µM (Fig. 5 and 6). It indicates that addition of auxin (2,4-D) and cytokinin (thidiazuron) in the embryogenic calli medium are important for development of embryogenic calli of coffee explants. The role of auxin in embryogenesis of coffee tissues was through acidification of cytoplasm and cell wall (Kutshera 1994).

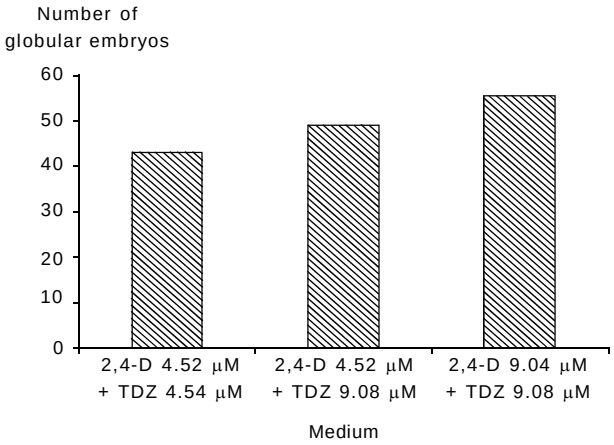

Fig 5. Number of globular embryos of arabica coffee through indirect somatic embryogenesis after four months cultured on regeneration medium. TDZ = thidiazuron.

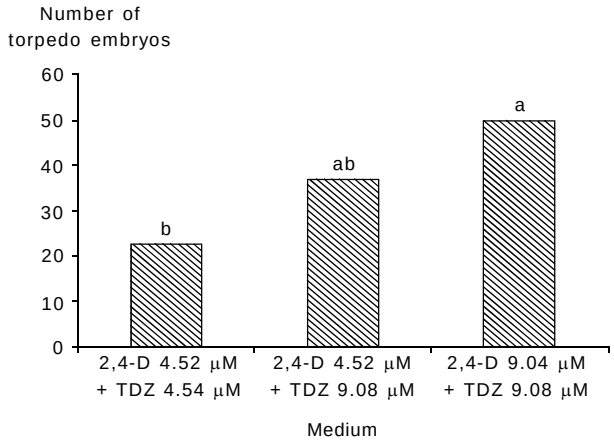

Fig 6. Number of torpedo embryos of arabica coffee through indirect somatic embryogenesis after eight months cultured on regeneration medium. The same letters on top of the bar indicate the treatment was not significantly different at α = 0,05. TDZ = thidiazuron.

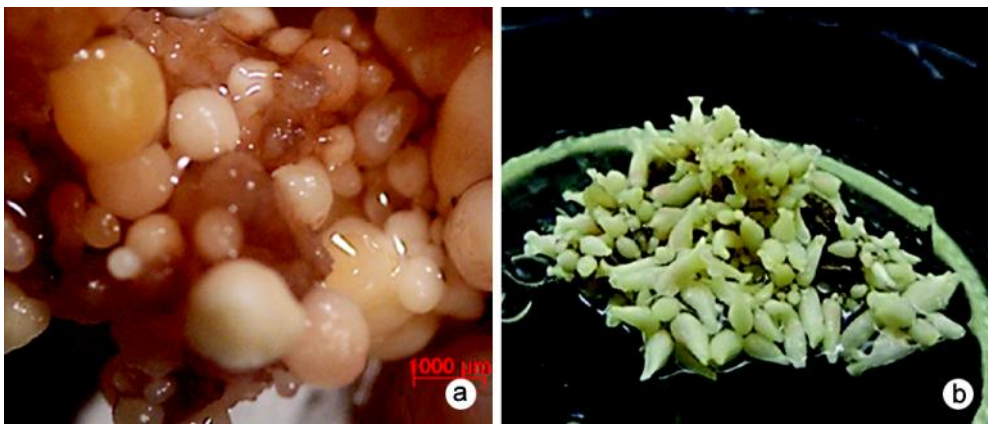

Fig. 4. Performance of arabica coffee calli by indirect somatic embryogenesis, (a) globular stage, (b) torpedo stage.

Direct Somatic Embryogenesis

In the direct somatic embryogenesis, globular embryo was developed from pre-embryo after two months in regeneration medium. The development of globular embryo was ununiform. Similar result was found by Gatica *et al*. (2008) and Oktavia *et al.* (2003) in the direct somatic embryogenesis of *C. arabica* using different plant growth regulators. The developmental stages of the embryo from the globular, heart and elongated embryo to torpedo were presented in Figure 7b. Higher number of globular and torpedo embryos were formed in the regeneration medium containing 2,4-D (2.26 µM) + thidiazuron (9.08 µM) (Fig. 8 and 9). Increase in thidiazuran concentration increased the number of globular and torpedo embryos produced.

The result showed that somatic embryo cultures of arabica coffee were produced both through direct and indirect embryogenesis. The success of callus forma-

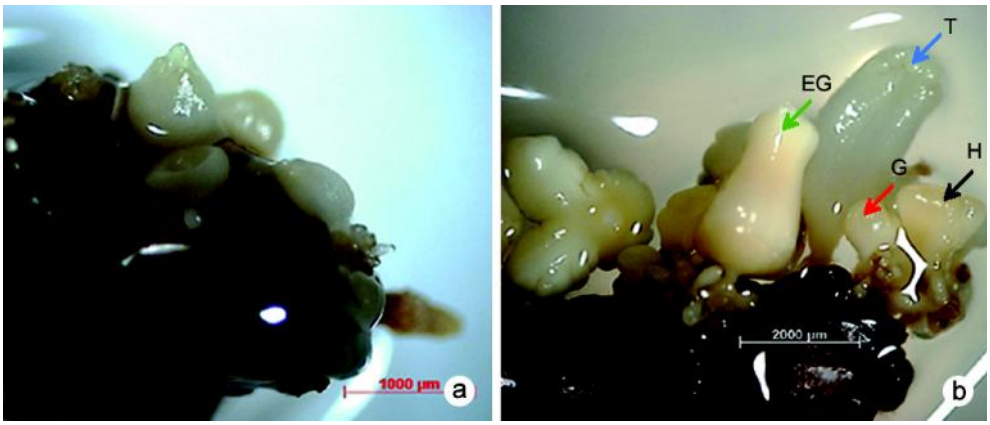

Fig. 7. Performance of different forms of somatic embryogenesis culture of arabica cofee by direct embryo-genesis; (a) globular forms of embryogenic cell cultures attached to the leaf tissue, (b) globular (G, red arrow), heart (H, black arrow), elongated embryo (EG, green arrow) and torpedo (T, blue arrow).

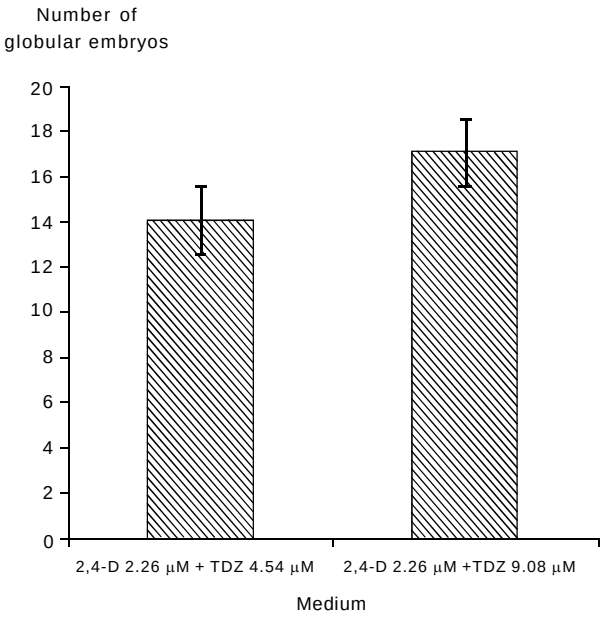

Fig. 8. Number of globular embryos of arabica coffee at three months after culture on regeneration medium. TDZ = thidiazuron.

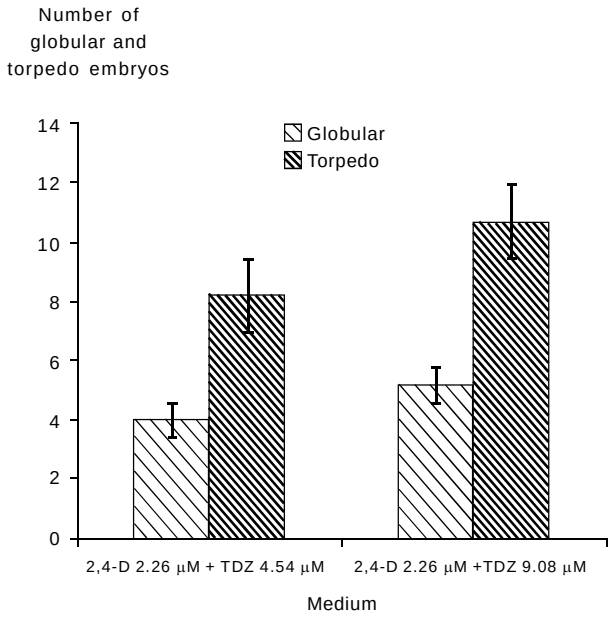

Fig. 9. Number of globular and torpedo embryos of arabica coffee at six months after culture on regeneration medium. TDZ = thidiazuron.

tion was influenced by the ratio of auxin and citokinin in the calus induction medium. Further studies are necessary to evaluate development of direct and indirect embriogenesis to form whole plant.

CONCLUSION

Somatic embryos of arabica coffee var. Kartika were successfully produced through direct and indirect somatic embryogenesis. The ratio and dosage of auxin (2,4-D) and citokinin (thidiazuron) was critial in success of callus formation. In the direct embryogenesis, combination of 2,4-D (2.26 µM) and thidiazuron (9.08 µM) produced more globular and torpedo embryos, whereas in the indirect embryogenesis addition of 2,4-D (9.04 µM) + thidiazuron (9.08 µM) produced more and uniform globular and torpedo embryos.

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REFERENCES

Albarra´N, J., B. Bertrand, M. Lartaud, and H. Etienne. 2005. Cycle characteristics in a temporary immersion bioreactor affect regeneration, morphology, water and mineral status of coffee (*Coffea arabica*) somatic embryos. Plant Cell Tiss. Org. Cult. 81: 27-36.

Arimarsetiowati, R. 2011. Pengaruh auksin 2,4-D dan sitokinin 2-ip terhadap pembentukan embriogenesis somatik langsung pada eksplan daun *Coffea arabica* L. Pelita Perkebunan 27(2): 68-77.

Calheiros, M.B.P., L.G.E. Vieira, and S.R.L. Fuentes.1994. Effects of exogenous polyamines on direct somatic embryogenesis in coffee. Bras. Physiol. Veg. 6(2): 109-114.

Dehayes, A. 2000. *In vitro* propagation in coffee and field performance of tissue culture plants. *In* N.S. Prakash, Y. Raghuramulu, and J. Devasia (Eds.). Proceedings of International Scientific Symposium on Coffee, Baangalore, India, 4 December 2000. pp. 47-52.

Etienne, H. 2005. Somatic embryogenesis protocol: Coffee (*Coffea arabica* L. and C. P.). *In* S.M. Jain and P.K. Gupta (Eds.). Protocol for Somatic Embryogenesis in Woody Plants. Springer, the Netherlands. pp 167-179.

Etienne, H., F. Anthony, S. Dussert, D. Fernandez, P. Lashermes, and B. Bertrand. 2002. Biotechnological applications for the improvement of coffee (*Coffea arabica* L.). In Vitro Cell. Dev. Biol. Plant. 381: 129-138.

Figueroa-Quiroz, F.R., C.F.J. Fuentes-Cerda, R. Rojas-Herrera, and V.M. Loyola-Vargas. 2002. Histological studies on the developmental stages and differentiation of two different somatic embryogenesis systems of *Coffea arabica*. Plant Cell Reports. 20: 1141-1149.

Gatica, A.M., G. Arrieta, A.M. Espinoza. 2008. Direct somatic embryogenesis in *Coffea arabica* L. cvs. Caturra and Catuaí: Effect of triacontanol, light condition, and medium consistency. Agronomía Costarricense 32(1): 139-147.

Gatica-Arias, A.M., G. Arrieta-Espinoza, and A.M.E. Esquivel. 2007. Plant regeneration via indirect somatic embryogenesis and optimisation of genetic transformation in *Coffea arabica* L. cvs. Caturra and Catuaí. Electronic Journal of Biotechnology. Pontificia Universidad Católica de Valparaíso-Chile 11(1): 1-12.

George, F.F. and Sherrington. 1984. Plant Propagation by Tissue Culture. Exegetic Ltd., England.

Giridhar, P., V. Kumar, E.P. Indu, Ravishankar, and A. Chandrasekar. 2004. Thidiazuron induced somatic embryogenesis in *Coffea arabica* L. and *Coffea canephora* P ex Fr. Acta Bot. Croat. 63(1): 25-33.

Ibrahim, M.S.D., Rr. S. Hartati, Rubiyo, A. Purwito, and Sudarsono. 2013. Embryogenic calli induction and regeneration potential of arabica coffee by 2,4-dichlorophenoxyacetic acid and 6-benzyladenine. Bul. Ristri 4(2): 91-96.

Ibrahim, M.S.D., Sudarsono, Rubiyo, dan Syafaruddin. 2012. Pengaruh komposisi media terhadap pembentukan kalus menuju induksi embrio somatik kopi arabika (*Coffea arabica*). Bul. Ristri 3(1): 13-22.

Kumar, V., N. Madhava, and G.A. Ravishankar. 2006. Developments in coffee biotechnology - *in vitro* plant propagation and crop improvement. Plant Cell Tiss. Org. Cult. 87: 49-65.

Kutschera, A. 1994. The current status of the acid-growth hypothesis. New Phytol.126: 549-569.

Nakamura, T., T. Taniguchi, and E. Maeda.1992. Studies on somatic embryogenesis of coffee by scanning electron microscope. Jap. J. Crop Sci. 61(3): 476-486.

Oktavia, F., Siswanto, A. Budiani, dan Sudarsono. 2003. Embriogenesis somatik langsung dan regenerasi planlet kopi arabika (C*offea arabica*) dari berbagai eksplan. Menara Perkebunan 71(2): 44-55.

Priyono. 2004. Kultur *in vitro* daun kopi untuk mengetahui kemampuan embriogenesis somatik beberapa varietas kopi. Pelita Perkebunan 20(3): 110-122.

Raghuramulu, Y., H.L. Sreenath, and P.K. Ramaiah. 1989. Regeneration of coffee plantlets through tissue culture techniques in India. J. Coffee Res. 19: 30-38.

Rai, V.R. and J. Mccomb. 2002. Direct somatic embryogenesis from mature embryos of sandalwood. Plant Cell Tiss. Org. Cult. 69: 65-70.

Ribeiro, T.O. and M.F. Carneiro. 1989. Micropropagation by nodal culture of cultivars Caturra, Geisha and Catimor regenerated *in vitro*. *In* ASIC Publishers (Eds.) 13th International Scientific Colloquium on Coffee. Paipa, Colombia, 21-25 August 1989, pp. 757-765.

Samson N.P., C. Campa, L. Le Gal, M. Noirot, G. Thomas, T.S. Lokeswari, and A. De. Kochko. 2006. Effect of primary culture medium composition on high frequency somatic embryogenesis in different *Coffea* species. Plant Cell Tiss. Org. Cult 86: 37-45.

Staritsky, G. 1970. Embryoid formation in callus tissues of coffee. Acta Bot. Neerl. 19: 509-514.

Tores, K.C. 1989. Tissue Culture Techniques for Horticultural Crops. Van Nostrand Reinhold, New York. 285 pp.

Van Boxtel, J. and M. Berthouly. 1996. High frequency somatic embryogenesis from coffee leaves: factors influencing embryogenesis, and subsequent proliferation and regeneration in liquid medium. Plant Cell Tiss. Org. Cult. 44(1): 7-17.

Von Arnold S., I. Sabala, P. Bozhkov, J. Dyachok, and L. Filonova. 2002. Developmental pathway of somatic embryogenesis. Plant Cell Tiss. Org. Cult. 69: 233-249.

Williams, E.G. and Maheswara. 1986. Somatic embryogenesis factors influencing coordinated behaviour of cells as on embryogenic group. Ann. Bot. 57: 443-462.